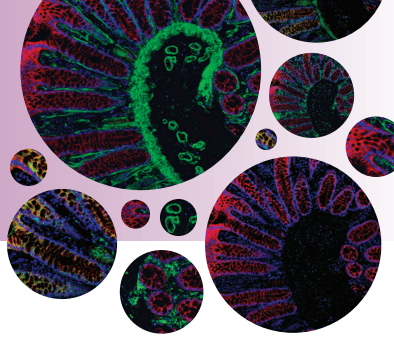
STANDARD BIOTOOLS

Application Note



Immuno-Oncology Panel Kits for Imaging Mass Cytometry

Introduction

Maxpar® Immuno-Oncology (IO) Panel Kits coupled with Imaging Mass Cytometry™ (IMC™) allow simultaneous high-plex detection of 40-plus markers. Bringing together high-parameter CyTOF® technology with imaging capability, the Hyperion™ and Hyperion+™ Imaging Systems provide unprecedented visualization of complex cellular phenotypes and their relationships in the context of cancer, immuno-oncology, and immune-mediated diseases. Maxpar panel kits for detection of human tumor-infiltrating lymphocytes, immune activation, and tissue architecture provide researchers with a richer, more insightful understanding of the cancer-related processes in the tumor microenvironment.

Objectives

- To illustrate the use of Maxpar Human Immuno-Oncology IMC Panel Kits to characterize specific cellular subpopulations in the context of the tissue microenvironment
- To demonstrate the power of high-plex imaging in examples of staining in different tissues, including normal and tumor samples

Advantages

- Ready to go—Get to results faster with these optimized IO panel kits. They include metal-labeled, pathologist-verified antibodies (25 µg of each antibody) with an intercalator tube for nucleic acid staining to accelerate your panel development.
- Modular—Mix and match these panel kits to answer different research questions. Also available is an IO combination panel kit that includes the markers of all three panel kits.
- Expandable—The Maxpar IO panel kits can be expanded with a range of additional pathologist-verified antibodies from our catalog or with Maxpar OnDemand™ antibodies, allowing custom panel development.

Human FFPE tonsil positive control tissue

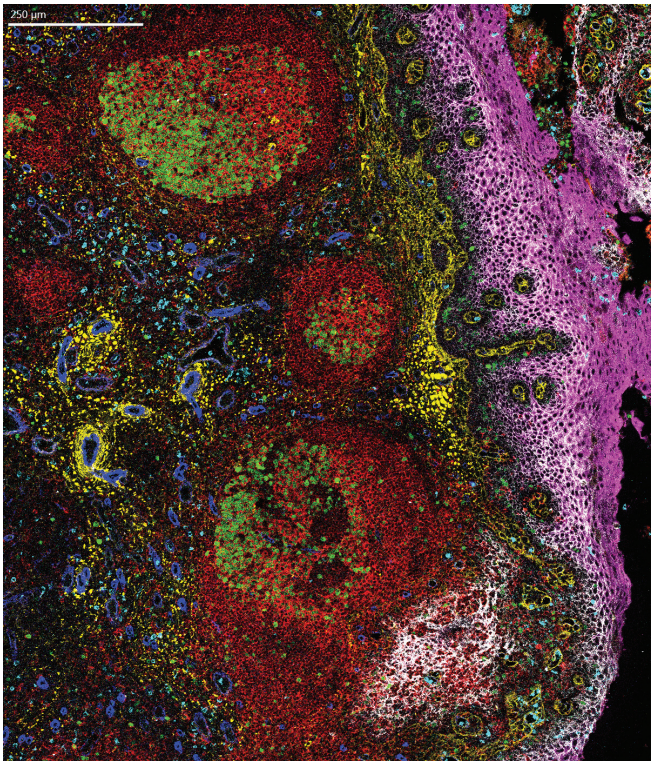


Figure 1. Human formalin-fixed, paraffin-embedded (FFPE) tonsil positive control tissue stained with the Maxpar Human Immuno-Oncology IMC Panel Kit. A composite of CD20 (red), alpha-smooth muscle actin (aSMA, blue), pan-keratin (magenta), Ki-67 (green), collagen 1 (yellow), granzyme B (cyan), and E- cadherin (white). Scale bar = 250 µm.

The Immuno-Oncology IMC Panel Kit

The Maxpar Human Immuno-Oncology IMC Panel Kit (Table 1) combines the Maxpar Human Tumor-Infiltrating Lymphocytes IMC Panel Kit, Maxpar Human Immune Activation IMC Panel Kit, and Maxpar Human Tissue Architecture IMC Panel Kit. This 18-plex kit enables interrogation of the tumor microenvironment including activated tumor-infiltrating lymphocytes and cancer cells in FFPE tissue sections using IMC.

Rationale

Each panel is designed with flexibility and versatility in mind so researchers can add custom antibodies in open channels or combine panels for deeper analysis.

Optimization

In the following experiment, FFPE normal human tonsil samples were stained with optimal antibody concentrations for all three panels. This enabled simultaneous identification of several different subpopulations of cells, their activation and cell cycle states, and their positional relationships. To further check for optimal staining beyond the positive control tonsil samples, the same concentration of antibody was applied to invasive ductal breast carcinoma samples. FFPE placenta was used to confirm PD-L1 staining in addition to tonsil.

Imaging

Samples are acquired with the Hyperion Imaging System and visualized on MCD™ Viewer, data processing software that allows users to visualize, review, and export Imaging Mass Cytometry data.

	Metal	Marker	Clone	Subpopulation	Recommended Dilution
Maxpar Human Tissue Architecture IMC Panel Kit	141Pr	Alpha-smooth muscle actin	1A4	Myofibroblasts, pericytes	1:400
	169Tm	Collagen 1	Polyclonal	Connective tissue	1:400
	158Gd	E-cadherin	24E10	Epithelial cells	1:400
	176Yb*	Histone H3	D1H2	Nucleated cells	1:400
	143Nd	Vimentin	D21H3	Mesenchymal cells	1:300
Maxpar Human Immune Activation IMC Panel Kit	167Er	Granzyme B	EPR20129-217	NK and T cells	1:100
	168Er	Ki-67	B56	Cycling cells	1:50
	165Ho	PD-1	EPR4877(2)	T follicular helper cells, activated T cells	1:50
	150Nd	PD-L1	SP142	Activated macrophages, dendritic cells, T and B cells	1:50
Maxpar Human Tumor-Infiltrating Lymphocytes IMC Panel Kit	161Dy	CD20	H1	B cells	1:600
	170Er	CD3	Polyclonal	T cells	1:50
	156Gd	CD4	EPR6855	Helper T cells	1:50
	173Yb	CD45RO	UCHL1	Memory lymphocytes	1:400
	159Tb	CD68	KP1	Dendritic cells, macrophage/ monocytes, granulocytes	1:600
	162Dy	CD8a	CD8/144B	Cytotoxic T cells	1:50
	155Gd	FoxP3	PCH101‡	T regulatory cells	1:50
	148Nd	Pan-keratin	C11	Keratinocytes	1:200
	191Ir/193Ir	Nucleic acid†	N/A	Nucleated cells	1:2000

Table 1. Maxpar IO IMC panel kits were optimized on normal tonsil. All stock antibodies are 25 µg/vial, concentration 0.5 mg/mL.

*The anti-histone H3 (D1H2) metal was changed from 171Yb to 176Yb to add flexibility for additional markers in neighboring channels.

†The nucleic acid stain iridium 191/193 stock is 500 µM, staining nucleic acid (DNA) for single-cell segmentation, and is included in each individual kit.

‡Due to an antibody supply constraint we have updated the FoxP3 clone from 236A/E7 to PCH101.

Application: FFPE Normal Human Tonsil (Positive Control)

Tissue architecture of tonsil

Tonsils are secondary lymphoid organs, which are sites of immune activation in the event of invasion by foreign organisms. They are composed of many lymphoid follicles encased by a layer of squamous epithelium. These follicles contain immune cells and develop into germinal centers upon immune activation. With a combination of structural and immunological markers, the tissue architecture of a lymphoid follicle and the surrounding epithelium can be examined at single-cell resolution.

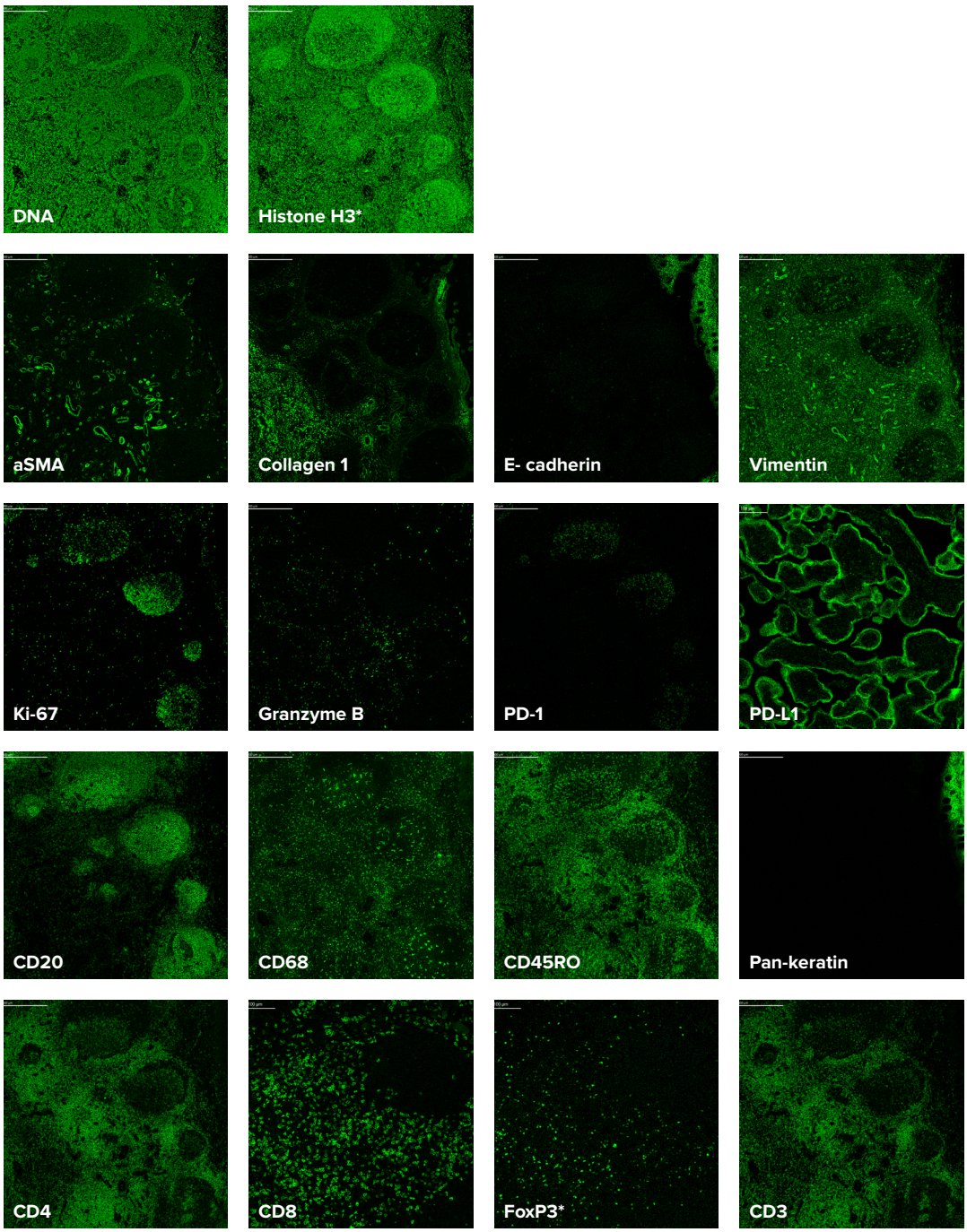


Figure 2. FFPE normal human tonsil and placenta samples examined by IMC. Each marker is shown individually in MCD Viewer, with gamma settings at 1 and thresholding the same. Pseudocolors can be applied to each channel to make composite images. PD-L1 stained shown in fresh placenta and all others shown in tonsil. Scale bar = 300 µm except 100 µm for PD-L1, CD8, and FoxP3.

*These images were acquired prior to the update of FoxP3 and histone H3 markers. See Table 1 for details.

Lymphocytes in the tonsil

The hematopoietic lineage marker CD45RO helps visualize the distribution of all immune cells in the tonsil tissue. Immune cells collect into aggregates known as follicles, denoted by the square box in Figure 3. Pan-keratin marks keratinocytes, which compose the outer epithelial layer of the tonsil, and in conjunction with E-cadherin, two layers of epithelium can be distinguished. An overlapping region in yellow illustrates anchoring of keratinocytes to the inner layer of tissue.

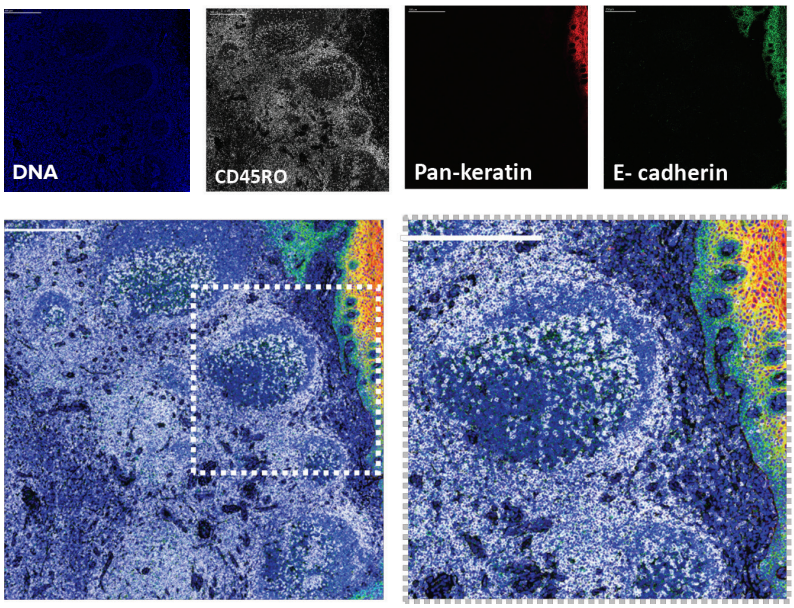


Figure 3. FFPE normal human tonsil samples examined by IMC with DNA (blue), CD45RO (white), pan-keratin (red), and E-cadherin (green). Each marker is shown individually (upper panels) and in composite (lower panels). Scale bars = 300 µm.

Localization of lymphocytes

CD20 and CD3 single-channel images in Figure 4 show organization of B and T cells, respectively.

Germinal centers develop from within follicles upon immune activation from encountering foreign antigen. Within a germinal center, B cells that have been activated by helper T cells migrate into “dark zones,” regions of rapid proliferation and differentiation.

In Figure 4, these regions are the zones with dense Ki-67 staining, in contrast to “light zones,” which are intra-follicular regions of sparse Ki-67 staining.

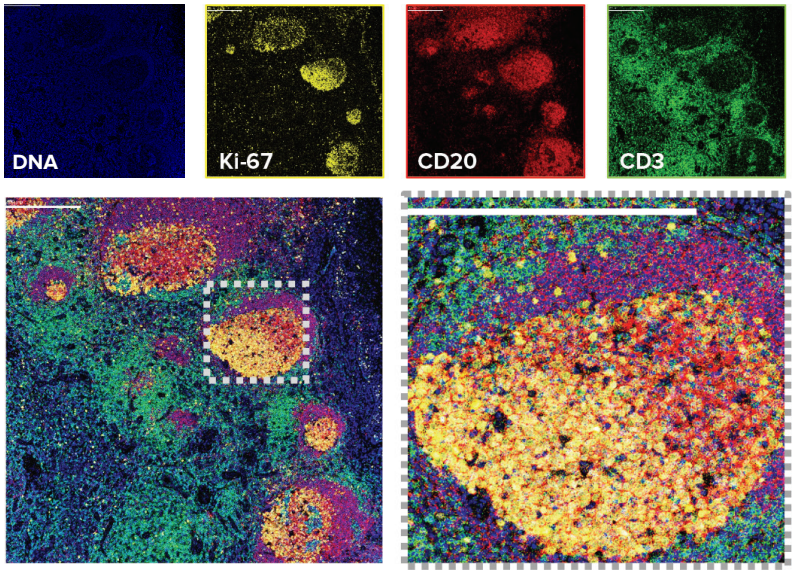


Figure 4. FFPE normal human tonsil examined by IMC with DNA (blue), Ki-67 (yellow), CD20 (red), and CD3 (green). Each marker is shown individually (upper panels) and in composite (lower panels). Scale bars = 300 µm.

Identification of rare populations

T follicular helper cells (TFH) are a subset of T cells responsible for activating B cells and promoting survival and differentiation into plasma cells. These populations are sparse when analyzed by single-cell suspension and require many markers to identify. In Figure 5, TFH populations are found within light zones of germinal centers and can be identified in this spatial context by membrane co-expression of CD4 and PD-1.

With the power of high-plex imaging by IMC, these rare cells can be readily distinguished with single-cell resolution.

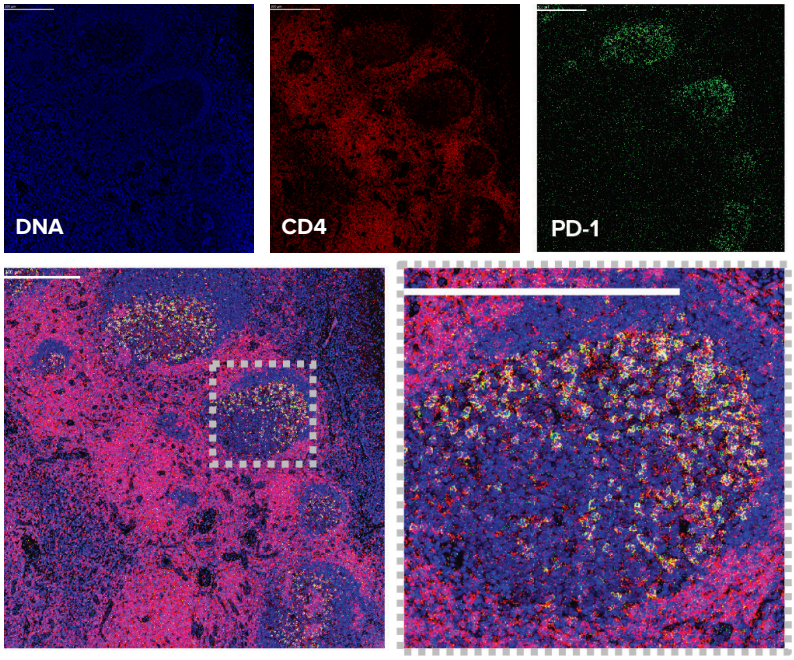


Figure 5. FFPE normal human tonsil samples examined by IMC with DNA (blue), CD4 (red), and PD-1 (green) markers. Each marker is shown individually (upper panels) and in composite (lower panels). Scale bars = 300 µm.

Application: FFPE Human Breast Carcinoma

Tissue architecture within the breast

Epithelial-to-mesenchymal transition (EMT) is a phenomenon observed in malignancies of epithelial origin. In these metastatic tumors the cells convert to a more stem cell-like phenotype to extravasate through vascular or lymphatic vessel walls and spread to distal sites.

Loss of E-cadherin is one of the identifiable hallmarks of this transition. Figure 6 shows a pocket of highly dense epithelium identified by E-cadherin and pan-keratin. Simultaneously, immune cell infiltration can be seen by expression of CD45RO (arrows).

Vimentin and aSMA are both used in identifying cells of mesenchymal origin. Figure 7B shows co-expression of aSMA$^+$/vimentin$^+$ pericytes and myofibroblasts, which are cells that provide support for blood vessels.

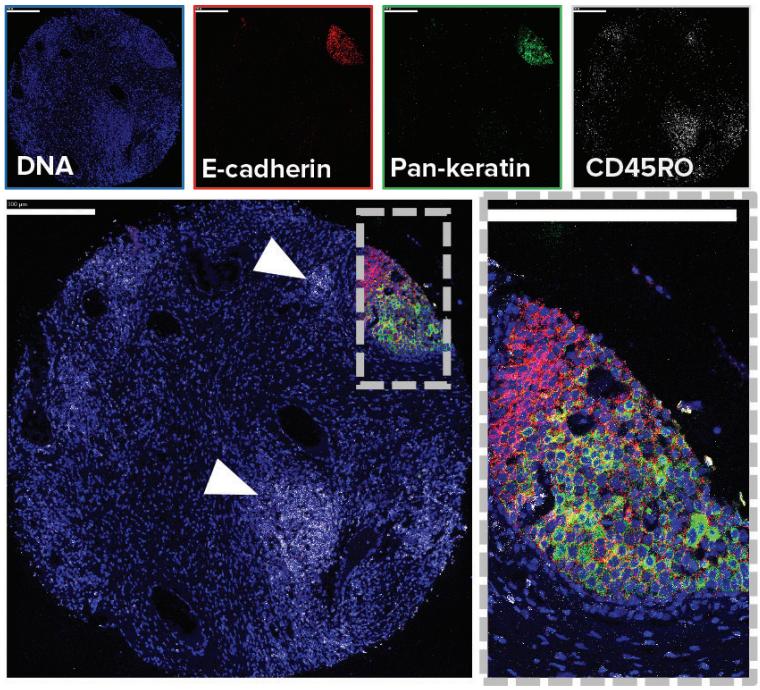


Figure 6. FFPE human breast carcinoma examined by IMC with DNA (blue), E-cadherin (red), pan-keratin (green), and CD45RO (white). A composite is shown (lower panels), with single-channel images in the upper panels. Scale bars = 300 µm.

Simultaneously, a subset of the cells identified as pan-keratin^{-}/ E-cadherin^{+} from Figure 6 is also Ki-67^{+}, suggesting that those cells are proliferative (Figure 7A).

Tumor-infiltrating lymphocytes within the breast

Examination of the T cell landscape identified CD8^{+} cytotoxic T cells interspersed throughout the CD45RO^{+} region. With the granzyme B marker from the Immune Activation Panel Kit, activated cytotoxic T cells can also be identified (Figure 8A white arrows).

Simultaneously, CD68^{+} monocyte-derived macrophages can be identified within the CD45^{+} regions. Using the granzyme B marker, activated CD68^{+} macrophages can be seen (Figures 8A and B).

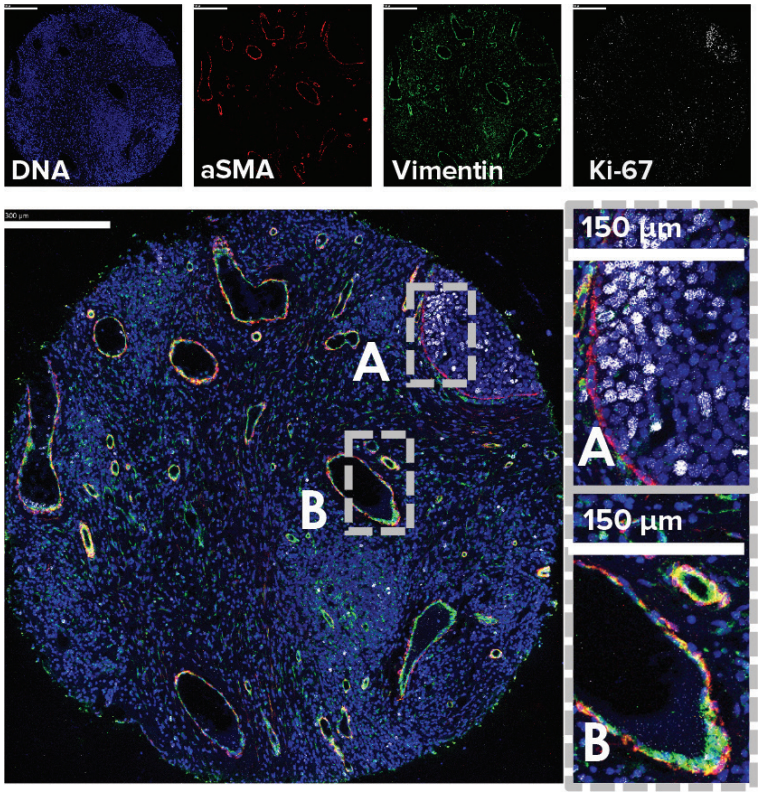


Figure 7. FFPE human breast carcinoma examined by IMC with DNA (blue), aSMA (red), vimentin (green), and Ki-67 (white). A composite is shown (lower panels), with single-channel images in the upper panels. Scale bars = 300 µm.

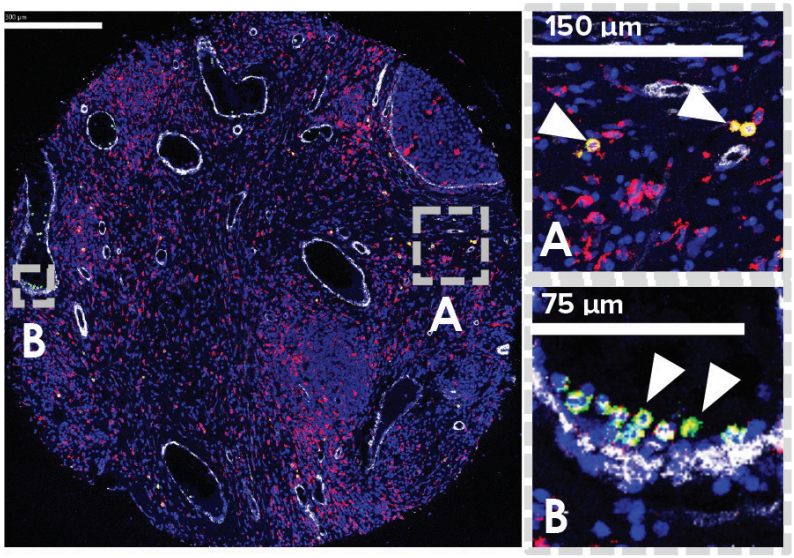


Figure 8. FFPE human breast carcinoma examined with DNA (blue), CD68 (red), granzyme B (green), and aSMA (white). A composite from single-channel images is shown. Scale bars = 300 µm.

Conclusion

IMC can provide highly multiplexed evaluations on tissue organization, enabling clarity into antigen density and receptor distribution across any given tissue section. IMC is a powerful tool that enables researchers to interrogate cellular phenotypes in the spatial context of the tissue microenvironment to gain valuable insights. These modular panel kits enhance the ability to probe tissues and to determine cellular relationships with high dimensionality.

Appendix: Materials and Method

Protocol

The Imaging Mass Cytometry Staining Protocol for FFPE Sections (PN 400322) can be downloaded from **standardbiotools.com**.

For detailed instructions on instrument operation, see the Hyperion and Hyperion+ Imaging Systems User Guide (PN 400311). Acquisition was performed using CyTOF Software v7.0, and pseudocolored images were generated using MCD Viewer v1.6.

Required reagents

Source	Product Name	Catalog Number
Standard BioTools	Maxpar® PBS	201058
	Cell-ID™ Intercalator-Ir	201192A
	Maxpar Water	201069
	Maxpar antibodies	Multiple
Third-Party Reagents		
Sigma-Aldrich®	M-xylene ReagentPlus®	185566-1L
Sigma-Aldrich	Anhydrous ethyl alcohol	676829
Agilent®	Antigen Retrieval Solution pH 9 (10x)	S236784-2
Thermo Scientific™	Triton™ X-100	85111
Sigma-Aldrich	Bovine Serum Albumin (BSA)	A3059
Newcomer Supply	FFPE human tonsil sections	3077A
US Biomax	FFPE breast cancer TMA	BR243u

Ordering Information

Panel	Catalog Number
Maxpar® Human Tumor-Infiltrating Lymphocytes IMC™ Panel Kit	201506
Maxpar Human Immune Activation IMC Panel Kit	201503
Maxpar Human Tissue Architecture IMC Panel Kit	201507
Maxpar Human Immuno-Oncology IMC Panel Kit	201508

If you are reordering, please note the changes in catalog number from 201502 to 201506 due to change in FoxP3 clone and from 201504 to 201507 due to optimization of histone H3. The complete kit catalog number 201505 is also updated to 201508 to reflect these changes.

Tips for Success

For the overall success of the protocol, we recommend the following best practices:

Sample integrity

To ensure best results, we recommend using FFPE samples that are freshly cut.

Antigen retrieval

To minimize the need for end user optimization, all IMC antibodies are tested using heat-induced antigen retrieval with a Tris-EDTA (pH 9) solution at 96 °C.

Antibody titration

Our panels were titrated in human FFPE tonsil samples. We recommend testing panels at different concentrations when imaging different tissues.

Antibody staining and signal intensity can vary greatly between different tissues (for example, spleen vs. lung), tissue types (for example, FFPE vs. fresh frozen), and method of antigen retrieval. All antibodies should be titrated on the tissue of interest to determine optimal staining concentration. A recommended concentration range is included on each antibody technical data sheet (TDS) for reference when determining an initial test dilution.

Panel design

IMC has the capacity for evaluation of far larger panels than multiplexed immunofluorescence or immunohistochemistry without requiring iterative or cyclic antibody staining. However, intelligent panel design is still a necessity for successful pilot experiments. Our customers are encouraged to reach out to their local Field Applications Specialist (FAS) for assistance when designing large panels.

Storage

Once staining has been done, keep samples in plastic slide holders inside a sealed bag to minimize humidity. High humidity can damage tissue integrity and decrease shelf life of stained sample slides.

Resources

To help assess antibody staining in the tissue of interest we recommend consulting the Human Protein Atlas (**proteinatlas.org**) in conjunction with GeneCards®: The Human Gene Database (**genecards.org**) for correct protein nomenclature.

Learn more at **standardbiotools.com**

Or contact: **tech.support@fluidigm.com**

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